Effectivity of Different Methods for the Extraction and Purification of IgY

Christine Schwarzkopf and Bernhard Thiele[1]

Bundesinstitut für gesundheitlichen Verbraucherschutz und Veterinärmedizin, D-Berlin, [1]Transplantationsimmunologie, D-Kaiserslautern

Summary

Extraction of IgY from the egg yolk of immunised laying hens differs from the conventional way of antiserum preparation following blood sampling or exsanguination also with regard to the preparation technique. Six methods of IgY preparation were studied comparatively. As a result, the ammonium sulfate precipitation proved to be the most environmentally compatible and economical one. The advantage of this method becomes particularly obvious when the method is followed by affinity chromatography.

Zusammenfassung: Effektivität verschiedener IgY-Extraktions- und Reinigungsmethoden.

Die Extraktion von IgY aus dem Eidotter immunisierter Hennen unterscheidet sich auch präparationstechnisch vom konventionellen Weg der Antiserumgewinnung nach Blutentnahme oder Entbluten. Von den hierzu vergleichend untersuchten sechs Extraktionsverfahren für IgY erwies sich mit der Ammoniumsulfatpräzipitation das umweltschonenste Verfahren gleichzeitig als das wirtschaftlichste. Dessen Vorzüge kommen besonders in der Kombination mit einer sich anschließenden Affinitätschromatographie zum Tragen.

Keywords: IgY, extraction, purification, precipitation

1 Introduction

Although there is now no substantial doubt about the immunological characteristics of IgY and the aspects of animal welfare associated with this kind of antibody production, it is still not widely used. One of the reasons for this consists in a lack of laboratory experience with the processing of egg yolk for the extraction of IgY. The methods used for the purification of mammalian immunoglobulins cannot be automatically applied to egg yolk and IgY. Also, there is a low acceptance of easy to use but expensive commercial extraction systems for the production of IgY. Beyond this, experience is missing concerning yield, purity, reproducibility, practicability, required manpower, and exact costs of these methods that may be used for purification. Likewise, studies are still missing which would compare the different methods by the above criteria.

A variety of methods for the extraction of IgY have been published recently. Six of the most commonly used have been examined in the present study. These were the dextran sulfate precipitation according to Jensenius (1981), the dextran sulfate precipitation according to Burstein and Samaille (1959), the PEG precipitation according to Polson (1980), the precipitation with PEG and ethanol according to Polson (1985), the ammonium sulphate precipitation (Wallmann and Staak, unpublished), and a precipitation with ammonium sulfate and octanoic acid according to McKinney and Parkinson (1987). None of them requires organic solvents except for the PEG/ethanol extraction.

2 Animals, materials, and methods

To obtain species-specific antibodies, layers of White Leghorn line were immunised by subcutaneous application of purified rabbit IgG, using 0.5 ml FCA per immunisation. The amounts of the target antigen were 5 mg for primary immunisation and 2.5 mg each for the booster injections (at day14 and 28, respectively).

2.1 Preparation of egg yolk anti bodies

After cracking the eggs, separating egg yolk and egg white, yolks were carefully rolled on filter paper until all egg white residues on the yolk membrane had vanished. This membrane was then perforated so that the yolk (10-15 ml) could flow from the filter paper directly into a small Erlenmeyer flask. Depending on the method of extraction, the corresponding dilution buffer was chosen. After dilution with double-distilled water, the specific IgY titre could be determined immediately by ELISA. Until use, the egg yolks were stored in a dilution of 1:5 with double-distilled water, at –20°C.

2.2 Determination of titre and binding activity

An indirect ELISA was used for determination of the titre directly from the yolk diluted with double-distilled water. Microtiter plates were coated with the respective target antigen (10 µg/ml carbonate buffer, pH 9.6) and, after repeated washings, incubated with an egg yolk dilution series from 1:16 to 1:32 000, followed by further washing cycles, final prior to an incubation with peroxidase-labelled rabbit anti-chicken antibody (Nordic, Tilburg, Netherlands), and development with ABTS® (Boehringer, Mannheim, Germany).

For comparison of the different extraction methods with regard to yield, purity, technological expenditure and ecological impact (table 1), all extracts were adjusted to a volume of 6 ml each (extraction volume) and tested for specific activity, again in the indirect ELISA.

Table 1: Methods of IgY extraction

Pool of egg yolks (40 ml each)					
$(NH_4)_2SO_4$	$(NH_4)_2SO_4$ + octanoic acid	DxS (1959)	DxS (1981)	PEG (1980)	PEG (1985)
		Dilution with:			
Double-distilled water	Acetate buffer	TBS	TBS	PBS	PBS
Freeze + thaw Centrifugation Supernatant + $(NH_4)_2SO_4$	+ octanoic acid	+ DxS + $CaCl_2$	+ DxS + $CaCl_2$	Freeze + thaw Centrifugation Supernatant + PEG	Freeze + thaw Centrifugation Supernatant + PEG
Incubation Centrifugation	Incubation Centrifugation	Incubation Centrifugation	Incubation Centrifugation	Incubation Centrifugation	Incubation Centrifugation
Precipitate + $(NH_4)_2SO_4$	Supernatant + $(NH_4)_2SO_4$	Supernatant	Supernatant + NA_2SO_4	Supernatant + PEG	Supernatant + PEG
Incubation Centrifugation	Incubation Centrifugation		Incubation Centrifugation	Incubation Centrifugation	Incubation Centrifugation
Precipitate + PBS	Precipitate + PBS		Precipitate + TBS	Precipitate + PBS + PEG	Precipitate + PBS + PEG
			Centrifugation	Incubation Centrifugation	Incubation Centrifugation
			Supernatant + Na_2SO_4		Precipitate + PBS + Ethanol
			Centrifugation		Centrifugation
			Precipitate + PBS	Precipitate + PBS	Precipitate + PBS
Dialysis + concentration					
Filtration (0.2 µm) + preservation					

2.3 Affinity chromatography

For this purpose, 10 mg of the respective target antigen per ml of gel bed volume were used for binding to CNBr-activated Sepharose 4B (Pharmacia, Freiburg)). Finally the specific IgY was eluted with glycin-HCl pH 2.3.

2.4 Determination of IgY concentration

The protein content of the products obtained by the different methods was determined through the Biuret method according to Weichselbaum (1946) and at low concentrations (< 1mg/ml), according to Bradford (1976). In both types of examination, bovine serum albumin (BSA) served as reference substance.

2.5 Cellulose acetate film electrophoresis

Electrophoresis was performed on an Olympus Hite System 300 (Olympus Optical, Hamburg, Germany) on a pretreated cellulose acetate microfilm. The protein bands were stained with Ponceau red S (Grabner et al., 1970). Optical density of the individual protein fractions was measured at 520 nm.

3 Results

The measure of the IgY content of the extracted protein given in table 2 results from protein determination and cellulose acetate film electrophoresis of the extract. The amount of specific IgY was determined from the protein concentration of the pure IgY fraction obtained from affinity chromatographic purification.

3.1 Yield

A direct comparison of the different methods of extraction revealed that the highest IgY yields could be obtained

by the ammonium sulfate method while the classical dextran sulfate method, the dextran sulfate method according to Burstein and Samaille and the PEG-ethanol method all led to substantially lower yields (table 2).

3.2 Purity

Only the dextran sulfate- and PEG/ethanol extraction can be regarded as highly purifying methods (table 2). However, this is less important if affinity-purified antibodies are required.

4 Discussion

4.1 Evaluation of the different methods of extraction and purification with regard to different purposes and conditions of use

For a comparison of different extraction methods, the respective criteria of evaluation were weighted according to criteria such as laboratory standard, staff costs, material costs, energy consumption and ecological balance as well as the type of product. With regard to working hours and staff costs, the differences between developing and industrialised countries will become evident. This is similarily true for cost factors such as laboratory equipment and expenditure on consumables. Although practice still differs, the criteria and requirements for ecological compatibility should be the same for all methods. On the other hand, the desired use of the IgY produced may decisively influence the choice of the extraction method. Thus, conjugation with peroxidase requires chromatographically pure antibody while primary antibodies for immunohistochemistry are mostly not subject to such purity requirements. Generally, a high content of foreign protein (ovalbumin, enzymes) is not desirable.

To permit weighting of conditions and requirements the criteria of yield, expenditure in terms of time and material and also ecological impact were rated by a score of 1 – 10 points, a score of „10" standing for a maximum yield, expenditure etc. The respective extraction methods are characterised by the ratio between yield score and expenditure score (table 3). Yield is evaluated by criteria such as absolute yield (less than purity), expenditure in terms of time by the utilisation of labour (or walk away capability, respectively), expenditure in terms of material by costs of reagents (very high for dextran sulfate) as well as apparatus required and energy consumption (number of centrifugation steps necessary), ecological impact by the requirement of environmentally polluting chemicals, materials and energy. It should be pointed out, that only those methods have been considered here which do not require organic solvents such as acetone and propandiol.

The comparison of the methods using ammonium sulfate, octanoic acid plus ammonium sulfate, dextran sulfate, PEG, and PEG plus ethanol revealed that the mild salt precipitation with ammonium sulfate was the most favourable due to its relatively high yield, low expenditure, and low environmental impact. The degree of purity which can be achieved only by methods with a high expenditure in terms of material and time becomes less important because affinity chromatography applied as a final step will result in a high-purity end product.

Even after a strong modification of weighting the different criteria, the ammonium sulfate precipitation will remain the method of choice, as a rule. This applies to conditions of high labour costs and high ecological standards (industrialised countries) as well as to conditions of a low budget and a high availability of labour at low costs. Also, under such aspects the most economic method has proved to be the ecologically most favourable one.

A method propagated by Akita and Nakai (1992, 1993) also known as „water dilution method" is comparable to the mild salt precipitation method used in the present study. According to the authors, the yield obtained with that

Table 2: Comparison of different methods of IgY extraction

Method	Extraction volume (adjusted)	Protein yield Total protein content in % of extraction volume (w/v)	IgY yield Total IgY content of extraction volume in mg Purity in % of total protein	Activity (after extraction, prior to chromatography)
Ammonium sulfate	6 ml	11.4 %	445 mg 65 %	1: 128 000
Octanoic acid and ammonium sulfate	6 ml	1.8 %	54 mg 54 %	1: 8 000
DxS (Jensenius)	6 ml	4.7 %	278 mg 98 %	1: 64 000
DxS (Burstein and Samaille)	6 ml	13.6 %	326 mg 43 %	1: 128 000
PEG	6 ml	0.7 %	32 mg 77 %	1: 2 000
PEG and ethanol	6 ml	4.3 %	248 mg 96 %	1: 32 000

For all methods, the resulting content of specific IgY was between 3 and 3.5 % of total IgY.

Table 3: Rating of the different IgY extraction methods

Method	Yield (total IgY)	Time consumption	Costs (material; equipment; energy)	Environmental impact (incl. energy)	Final Score [yield/(costs+ time + environmental impact)]
Ammonium sulfate	10	4	4	4	0.83
Octanoic acid and ammonium sulfate	1	3	4	4	0.09
DxS (Jensenius, 1981)	6	8	8	8	0.25
DxS (Burstein & Samaille, 1959)	7	3	6	3	0.59
PEG (Polson, 1980)	1	6	6	5	0.06
PEG and ethanol (Polson, 1985)	5	8	8	7	0.22

method has been clearly superior to those yields obtained by other methods (dextran sulfate, xanthane, PEG/ethanol). Akita and Nakai used sodium sulfate in various concentrations for the precipitation of IgY in an aqueous medium in analogy to the two methods of ammonium sulfate concentration described here. The degree of IgY purity in the ovalbumin-free solution can be enhanced by final gel filtration or ion exchange chromatography. An exclusive use of ion exchangers for IgY preparation has also been described (Schade et al., 1994; Fichtali et al., 1993). If, however, specific IgY, purified by affinity chromatography is desired as the final product, solid phase absorption of this type becomes superfluous and therefore also the requirement of a high degree of purity for the initially extracted antibody.

With regard to the dextran sulfate method, Akita and Nakai (1993) refer to Jensenius et al. (1981) who stated that the decisive factor for this method was the exact amount of dextran sulfate and calcium chloride to be added in the first step. A too low amount would impede the subsequent salt precipitation with sodium sulfate. In the authors' experience, costs of reagents and the time required seem to limit the use of the dextran sulfate method.

Knowing either the efficiency of the respective method of purification or the kinetics of antibody production of hens after immunisation, the potential for IgY production as compared to that of mammalian antibody may be evaluated. Using ammonium sulfate, 110 mg protein having an IgY purity degree of 65 %, corresponding to 72 mg IgY, can be isolated from one egg (see data from table 2: 6 ml extract from 4 eggs; 440 mg/4 = 110 mg). About 2.5 mg of this amount consists of specific IgY, corresponding to 3–3.5 % of total IgY. If more than 10 eggs are to be processed at a time, the relative loss of IgY will be reduced resulting in higher yields of up to 3.5 mg of specific IgY per egg, corresponding to 5 % of total IgY. These data are in accordance with those of Gassmann et al. (1990a, 1990b) who obtained 2.1 mg specific IgY per egg, corresponding to 3.25 % of total IgY after preparation.

From rabbits, a single amount of 10-50 mg specific immunoglobulin can be obtained by exsanguination after day 35 of regular immunisation. In contrast to that, a yield of about 2-3.5 mg specific IgY per egg can be obtained daily from approximately day 30-50 after primary immunisation. Thus, almost unlimited amounts of antibody of high specificity would be available for practical use (fig. 1).

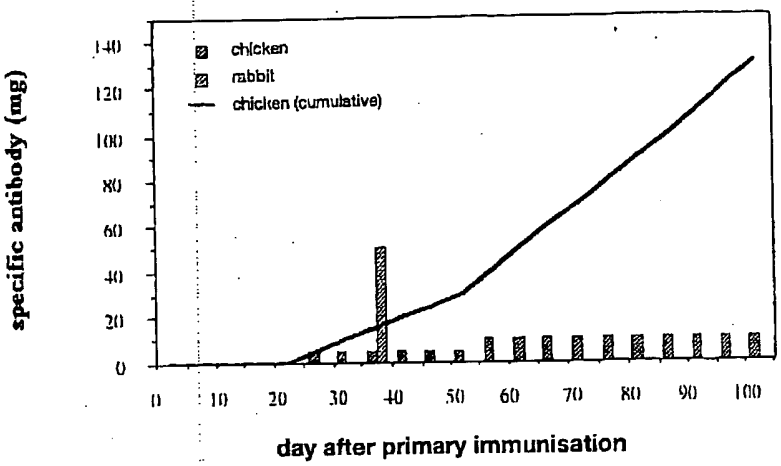

Figure 1: Amount of antibodies that can be obtained from chickens (IgY; egg yolk antibodies) and rabbits (IgG, serum antibodies)

References

Akita, E. M. and Nakai, S. (1992). Immunoglobulins from egg yolk: isolation and purification. *J. Food Sci. 57*, 629–634.

Akita, E. M. and Nakai, S. (1993). Comparison of four purification methods for the production of immunoglobulins from eggs laid by hens immunized with an enterotoxigenic E.coli strain. *J. Immunol. Methods 160*, 207–214.

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Bioch. 72*, 248–252.

Burstein, M. et Samaille, J. (1959). Nouvelle methode de seperation et de dosage

des lipoproteins de faible densite. *Ann. Biol. Clin. XVII*, 23–34.
Fichtali, J., Charter, E. A., Lo, K. V. and S. Nakai, S. (1993). Purification of antibodies from industrially separated egg yolk. *J. Food Sci. 58*, 1282–1285.
Gassmann, M., Thömmes, P., Weiser, T. and Hübscher, U. (1990a). Efficient production of chicken egg yolk antibodies against a conserved mammalian protein. *FASEB J. 4*, 2528–2532.
Gassmann, M., Weiser, T., Thömmes, P. und Hübscher, U. (1990b). Das Hühnerei als Lieferant polyklonaler Antikörper. *Schweiz. Arch. Tierheilkd. 132*, 289–294.
Grabner, W., Bergner, D. und Wermuthj, G. (1970). Mikrozonenelektrophorese auf Membranfolien. *Ärztl. Lab. 16*, 193–201.
Jensenius, J. C., Andersen,I., Hau, J., Crone, M. and Koch, C. (1981). Eggs: conveniently packaged antibodies. Methods for purification of yolk IgG. *J. Immunol. Methods 46*, 63–68.
McKinney, M. M. and Parkinson, A. (1987). A simple, non-chromatographic procedure to purify immunoglobulins from serum and ascites fluid. *J. Immunol. Methods 96*, 271–278.
Polson, A., Wechmar, M. B. von and Regenmortel, M. H. V. van (1980). Isolation of viral IgY antibodies from yolks of immunized hens. *Immunol. Comm. 9*, 475–493.
Polson, A., Coetzer, T, Kruger, J., Maltzahn, E. von und Merwe, K. J. van der (1985). Improvements in the isolation of IgY from the yolks of eggs laid by immunized hens. *Immun. Invest. 14*, 323–327.
Schade, R., Bürger, W., Schöneberg, T., Schniering, A., Schwarzkopf, C., Hlinak, A. und Kobilke, H. (1994). Avilre vitelline Antikörper (Dotterantikörper). *ALTEX 2*, 75–84
Weichselbaum, T. E. (1946). An accurate and rapid method for the determination of proteins in small amounts of blood serum and plasma. *Am. J. Clin. Pathol. 16*, 40–49.

Acknowledgement
This work was supported by BMBF (Bundesministerium für Forschung und Technologie), grant no. 0310125 B.

Corresponding address
Dr. Christine Schwarzkopf
Bundesinstitut für gesundheitlichen Verbraucherschutz und Veterinärmedizin (BgVV)
Diedersdorfer Weg 1
D-12277 Berlin